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# ELECTROPHORETIC ANALYSES OF PROTEINS AND ENZYMES IN *CULICOIDES VARIIPENNIS* (DIPTERA: CERATOPOGONIDAE)

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**Abstract**—1. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to study the changes that occur in the electrophoretic profile of whole body homogenates of *Culicoides variipennis* (Coquillett) during egg–adult morphogenesis.

2. Relative quantitative changes in particular bands during the course of insect development were observed.

3. Isoelectric focusing on polyacrylamide gels (IFPAG) was used to study the isozymes of aldehyde oxidase, non-specific esterases, α-glycerophosphate dehydrogenase, hexokinase, isocitrate dehydrogenase, malate dehydrogenase, malic enzyme, peptidase, phosphoglucoisomerase, phosphoglucomutase, 6-phosphogluconate dehydrogenase and triosephosphate isomerase.

4. Variability was evident in six of these enzyme systems.

## INTRODUCTION

Insect proteins have been separated and in some cases characterized by various workers. These studies have focused on the comparison of protein patterns for taxonomic purposes (Stephen, 1958; Halliday, 1975; Nunamaker and Wilson, 1981a; Nunamaker *et al.*, 1984, 1985), changes in protein composition during post-embryonic development (Loughton and West, 1965; Nunamaker and Wilson, 1981b, 1982), enzymatic activity (Laufer, 1960, 1961) and synthesis (Chen and Levenbook, 1966). Recently, electrophoretic techniques have been useful in the genetic characterization of insect vectors, primarily in various groups of mosquitoes (Ahmad *et al.*, 1978; Miles, 1979; Munstermann, 1979; Steiner *et al.*, 1982; Tabachnick *et al.*, 1984; Tabachnick and Wallis, 1985; Eldridge *et al.*, 1986) and ticks (Hilburn and Sattler, 1986).

*Culicoides variipennis* (Coquillett) is probably the most economically important species of biting midge in the United States due to its involvement in epizootic haemorrhagic disease of deer and the transmission of blue-tongue disease of sheep, cattle and ruminant wildlife (Jones, 1960; Luedke *et al.*, 1985). The variability of many morphological features of this midge was recognized many years ago when the species was first implicated in the transmission of blue-tongue. Wirth and Morris (1985) recommended use of the term "variipennis complex" until such time that the necessary biosystematic studies have been made to determine the genetic nature of the population structure over the entire geographic range of the species.

Although electrophoresis has been used in the recognition of species complexes in various animals (Selander *et al.*, 1971; Mahon, 1974), there have been no published reports of electrophoretic analyses of *C. variipennis*. To estimate the genetic variability within this taxonomic complex, it is necessary to determine whether isozymes can be readily detected in individual specimens of *C. variipennis*. Indeed, most previous reports of isozymes in insects have involved much larger species. Aphids, a notable exception, exhibit parthenogenesis, hence the genetically identical offspring from a single female can be "pooled" prior to electrophoresis.

The purposes of this investigation were: (1) to identify qualitative and/or quantitative changes in *C. variipennis* proteins during development from the egg through the adult stage and (2) to compare the quality of several enzyme assays of a laboratory colony of *C. variipennis*.

## MATERIALS AND METHODS

### *Experimental insects*

The *C. variipennis* specimens used in this study were from a colony that was established in 1957 and has been maintained in isolation and without the addition of field-collected flies (Jones, 1957). The insects were raised at 26.5°C and 40–60% relative humidity.

*Eggs.* Four milligrammes aliquants of random age eggs were analyzed for general proteins using isoelectric focusing on polyacrylamide gels (IFPAG).

*Larvae.* Due to their small size and because the rearing medium contained specimens of different ages, larvae could not be separated and collected according to their age. Consequently, larvae were sorted by size using USA Standard Testing Sieves* (Table 1). A 16 mg aliquant of each of four groups (sizes) of larvae (L-1, L-2, L-3 and L-4) was rinsed in distilled water and subdivided into 4 mg samples. This was done so that the same weights from each of the

*Mention of a proprietary product does not constitute an endorsement by the USDA.

Table 1. Life stages of *Culicoides variipennis* analyzed by SDS-PAGE

| Life stage | Reference code | Sieve size used to collect specimens | Age of specimens |
|---|---|---|---|
| Larva | L-1 | No. 140 (106 mm$^2$) | 2nd instar |
| Larva | L-2 | No. 100 (150 mm$^2$) | 3rd instar |
| Larva | L-3 | No. 80 (180 mm$^2$) | 3rd instar |
| Larva | L-4 | No. 70 (212 mm$^2$) | 4th instar |
| Pupa | P-1 | — | 2 ± 2 hr |
| Pupa | P-2 | — | 12 ± 2 hr |
| Pupa | P-3 | — | 24 ± 2 hr |
| Pupa | P-4 | — | 36 ± 2 hr |
| Adult | A-1 | — | 2 ± 2 hr |
| Adult | A-2 | — | 36 ± 2 hr |
| Adult | A-3 | — | 58 ± 6 hr |
| Adult | A-4 | — | 78 ± 6 hr |

four groups (L-1, L-2, L-3 and L-4) could be compared electrophoretically (i.e. L-1 contained far more larvae than L-4, since the former were much younger and therefore smaller in size, but the total weight of each subgroup was 4 mg). Larvae were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

*Pupae.* Four ages of pupae were analyzed by SDS–PAGE (Table 1). Each 4 mg subgroup was processed the same as the larvae.

*Adults.* Four ages of adult female flies were analyzed by SDS–PAGE (Table 1). Each 4 mg subgroup was processed the same as the larvae. In addition, adult flies (24–48 hr) were analyzed for isozyme activity by using IFPAG.

### *SDS–PAGE*

The SDS–PAGE techniques used in our laboratory are similar to previously published methods and are very satisfactory for the study of protein components in *C. variipennis*. However, since certain modifications have been beneficial, we will describe our procedure in detail.

*Tank buffer.* 288 g glycine, 60.6 g Trizma® base, 1.95 l distilled $H_2O$, 50 ml 20% SDS (use lauryl sulphate). Dilute 1:5 with distilled $H_2O$.

*Running buffer.* 2.0 M Trizma® base, adjust pH to 9.1 with HCl at 25°C.

*Stacking buffer.* 0.5 M Trizma® base, adjust pH to 6.7 with HCl at 25°C.

*Acrylamide stock solution.* 40 g acrylamide, 1.1 g *N,N'*-methylene bis acrylamide (bis). Adjust to 100 ml with distilled $H_2O$.

*Ammonium persulphate solution.* 0.15 g ammonium persulphate per 10 ml distilled $H_2O$.

Separating Gel (makes two gels 12 cm high × 14 cm wide × 0.15 cm thick)—42 ml distilled $H_2O$, 18 ml acrylamide stock solution, 13.6 ml running buffer, 370 µl 20% SDS, 40 µl TEMED, 50 mg sodium bisulphite, 300 µl ammonium persulphate. Mix the first four ingredients together and stir rapidly for 30 sec, add TEMED, continue stirring for 30 sec, add sodium bisulphite and continue stirring 30 sec. Finally, slowly drip ammonium persulphate solution into the rapidly stirring mixture over a period of 15 sec. Quickly pipette into the gel mold and overlay with *n*-butanol. Allow to polymerize for at least 1 hr.

Stacking Gel (makes two gels 4 cm high × 14 cm wide × 0.15 cm thick)—9 ml distilled $H_2O$, 2.2 ml acrylamide stock solution, 1.67 ml stacking buffer, 60 µl 20% SDS, 25 µl TEMED, 35 mg sodium bisulphite, 200 µl ammonium persulphate solution. Mix as for separating gel.

### *Samples (SDS–PAGE)*

Each 4 mg insect sample of eggs, larvae, pupae, or adults was placed into a 0.2 ml tissue grinder (Wheaton®) containing 40 µl grinding buffer (25 ml 0.5 M Trizma® base, pH 6.7, 2 g SDS, 10 ml glycerol, 5 ml 2-mercaptoethanol, distilled $H_2O$ to 100 ml). Samples were homogenized and transferred into 1.5 ml centrifuge tubes containing 25 µl PSM (10 ml 20% SDS, 5 ml saturated sucrose, 0.04843 g Trizma base, 0.04 mg bromophenol blue, 0.5 g dithiothreitol; adjusted to pH 8.0 with HCl and add 20 ml distilled $H_2O$). Samples were then boiled for 2 min, centrifuged at 16,000 ***g*** for 30 min and frozen at −70°C prior to SDS–PAGE.

### *Electrophoresis*

Gels were run in pairs at 1000 V, 60 mA and 15 W. The gels were allowed to run for 3–3.5 hr at which time they were fixed for 40 min in fixer/destain (100 ml isopropanol and 50 ml acetic acid), stained for 3 hr in Coomassie Blue stain (200 ml acetic acid, 500 ml isopropanol, 1 g Coomassie Blue R-250), and destained in fixer/destain. To improve the level of destaining a Kim-Wipe® was added to the agitating destain solution to filter out the Coomassie Brilliant Blue. After destaining, the gels were photographed, then scanned with an LKB 2222 Laser Densitometer (absorbance = 633 nm).

### *IFPAG techniques and solutions*

*Acrylamide and bis stock solution.* Dissolve 29.1 g acrylamide and 0.9 g bis in 75 ml distilled $H_2O$. Stir until solution is clear, then adjust to 100 ml with distilled $H_2O$, and filter solution.

*Ammonium persulphate stock solution.* Dissolve 0.1 g ammonium persulphate in 1 ml distilled $H_2O$. The solution should be used when fresh.

*Gel.* The procedure for making one 0.5 mm thick polyacrylamide gel (T = 7.5%, C = 3%, pH range 3.5–9.5, 110 × 245 mm) is as follows—mix togther 6.25 ml acrylamide and bis stock solution, 1.90 ml LKB pH 3.5–9.5 Ampholine® carrier ampholytes, and 16.7 ml distilled $H_2O$. Deaerate 5 min in a Buchner flask, then add 175 µl ammonium persulphate stock solution and 25 µl TEMED. Mix gently by swirling and quickly pour into gel mould.

### *Electrode solutions for 3.5–9.5 pH range*

*Anode.* Mix 0.33 g aspartic acid (mol. wt 132) and 0.37 g glutamic acid (mol. wt 146), and adjust to 100 ml with distilled $H_2O$.

*Cathode.* Mix 13.2 ml ethylenediamine (mol. wt 60), 0.44 g arginine (mol. wt 174) and 0.40 g lysine (mol. wt 164), and adjust to 100 ml with distilled $H_2O$.

### *Samples (IFPAG)*

Four milligrammes aliquants of eggs or entire individual adult female flies were homogenized (in an ice bath) in 0.2 ml Wheaton® tissue grinders containing 5 µl distilled $H_2O$. The homogenate was centrifuged for 2 min at 16,000 ***g*** to remove particulate matter. 4 µl of homogenate was applied directly to the gel surface as a drop, approximately 1 cm from the cathode strip.

*Running conditions for 3.5–9.5 pH range.* Gels were run at 2000 V, 15 mA and 15 W for 90–120 min at 10°C. Immediately after IFPAG, gels to be stained for general proteins were fixed for 30 min in fixing solution (add 28.7 g trichloroacetic acid and 8.62 g sulphosalicylic acid to 250 ml distilled $H_2O$), washed for 5 min in destaining solution (mix 250 ml ethanol and 80 ml acetic acid and dilute to 1 l with distilled $H_2O$), and stained for 15 min at 60°C in staining solution (dissolve 0.46 g Coomassie Brilliant Blue R-250 in 400 ml destaining solution). Gels were destained in several changes of destaining solution and were photographed.

### *Enzymes*

Enzyme activity was demonstrated by incubating the gel in the appropriate staining solution immediately following IFPAG. The stain recipes were from Steiner and Joslyn (1979), Baker (1974) and Shaw and Prasad (1970) (Table 2).

Table 2. Enzymes detected in adult female *Culicoides variipennis*

| Enzyme | Source of stain recipe |
|---|---|
| Aldehyde oxidase (Aldox; EC 1.2.3.1) | Steiner and Joslyn, 1979 |
| Esterase (Est; EC 3.1.1.8) | Shaw and Prasad, 1970 |
| α-Glycerophosphate dehydrogenase (α-Gpdh; EC 1.1.1.8) | Steiner and Joslyn, 1979 |
| Hexokinase (Hk; EC 2.7.1.1) | Steiner and Joslyn, 1979 |
| Isocitrate dehydrogenase (Idh; EC 1.1.1.42) | Steiner and Joslyn, 1979 |
| Malate dehydrogenase (Mdh; EC 1.1.1.37) | Shaw and Prasad, 1970 |
| Malic enzyme (Me; EC 1.1.1.40) | Shaw and Prasad, 1970 |
| Peptidase (Pep; EC 3.4.11.11) | Baker, 1974 |
| Phosphoglucoisomerase (Pgi; EC 5.3.1.9) | Steiner and Joslyn, 1979 |
| Phosphoglucomutase (Pgm; EC 5.4.2.2) | Steiner and Joslyn, 1979 |
| 6-Phosphogluconate dehydrogenase (6-Pgdh; EC 1.1.1.43) | Steiner and Joslyn, 1979 |
| Triosephosphate isomerase (Tpi; EC 5.3.1.1) | Steiner and Joslyn, 1979 |

## RESULTS

The densitometric tracings of the Coomassie Blue–stained SDS gels are shown in Figs 1–4. The distances (in mm) of the bands from the bottom of the gel are depicted along the *X*-axis. It is evident from these data that changes occur in the electrophoretic profile of *C. variipennis* during egg–adult metamorphosis. Although many bands can be detected during all life stages, there are dramatic quantitative changes in particular bands during the course of development.

In region (a) (Figs 1–4), for example, a peak that is relatively constant in eggs and L-1 and L-2 larvae increases markedly in intensity beginning in L-3, reaches its maximum height during P-2, and then gradually decreases through A-4. Region (b), on the other hand, contains a peak that fluctuates moderately from egg through L-4, then increases slightly during P-1 and P-2, and then decreases through A-4 to approximately the same intensity as that observed during the egg and larval stages. In region (c), a peak that is relatively uniform during the egg and L-1 through L-3 stages increases dramatically during L-4 and P-1 then very gradually decreases from P-2 through A-1, then remains constant throughout the four ages of the adult stage. In region (d), a major peak that is not well defined during the egg stage gradually increases during L-1 through P-4 and then stays relatively constant throughout the adult stage.

The electrophoretic patterns of the 12 enzyme systems that could be detected in *C. variipennis* are

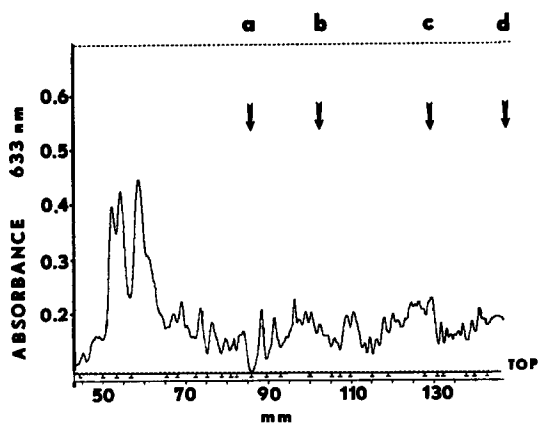


Fig. 1. Densitometric tracings (633 nm) of SDS gels containing egg protein components (mixed ages) from *Culicoides variipennis*. Regions a–d are discussed in the text.

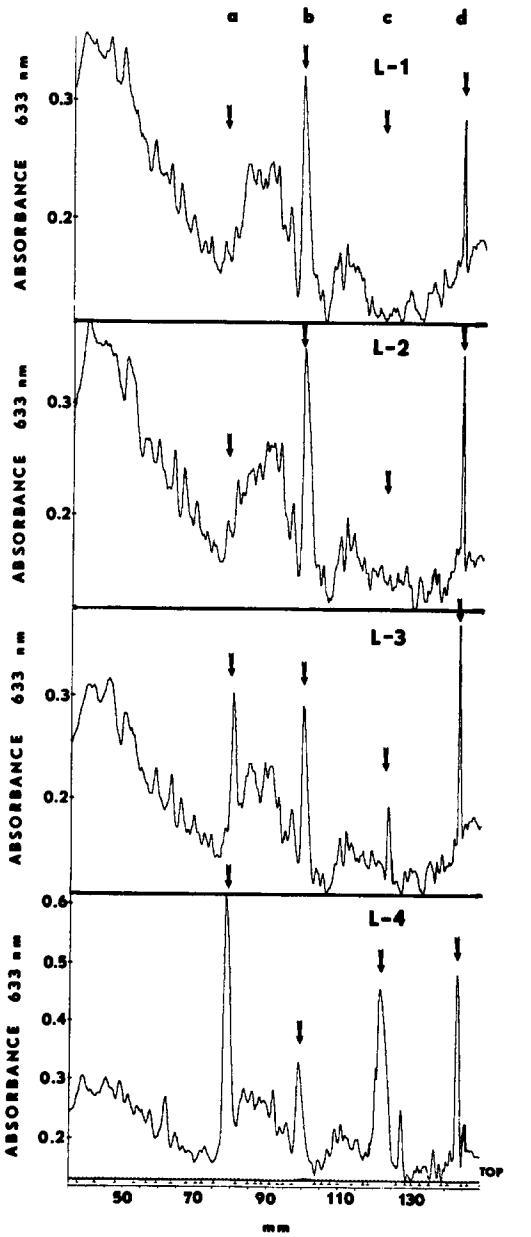


Fig. 2. Densitometric tracings (633 nm) of SDS gels containing larval protein components from *Culicoides variipennis*. L-1 = second instar, L-2 = early third instar, L-3 = late third instar, L-4 = fourth instar. Regions a–d are addressed in the text.

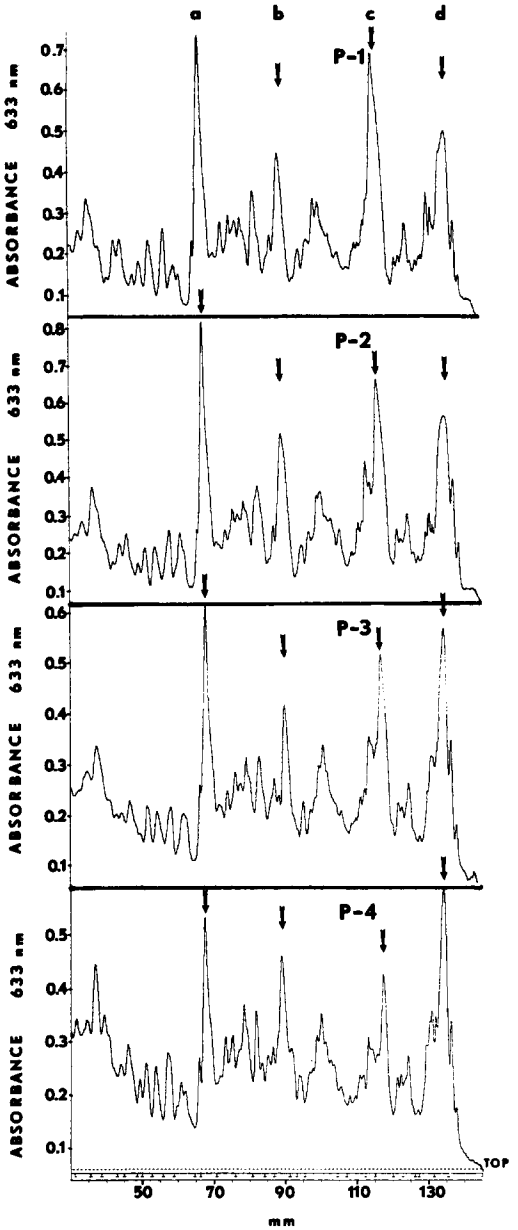


Fig. 3. Densitometric tracings (633 nm) of SDS gels containing pupal protein components from *Culicoides variipennis*. P-1 = 2 hr old, P-2 = 12 hr old, P-3 = 24 hr old, P-4 = 36 hr old. Regions a–d are addressed in the text.

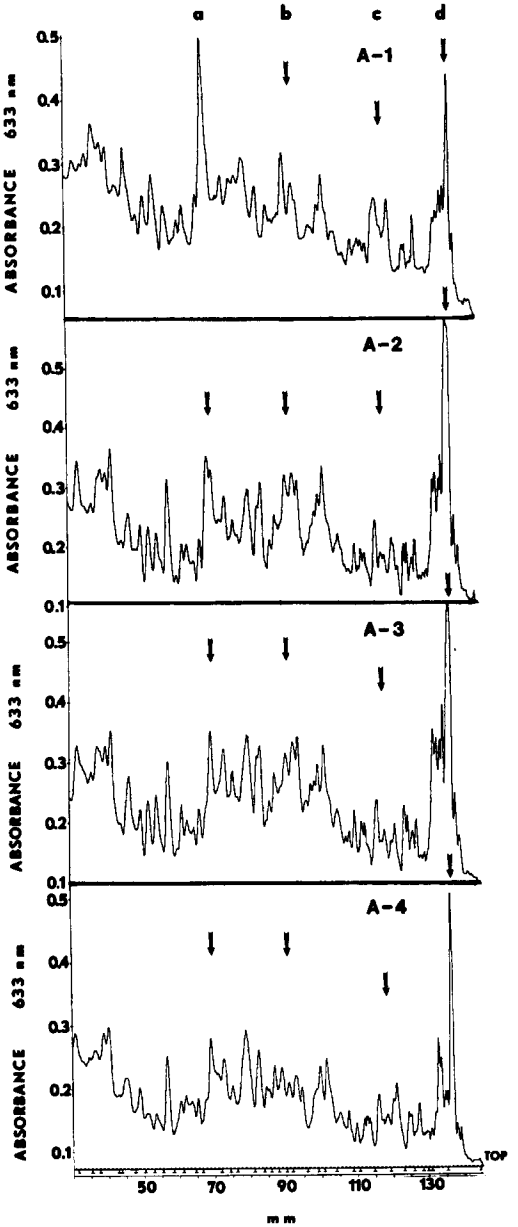


Fig. 4. Densitometric tracings (633 nm) of SDS gels containing adult protein components from *Culicoides variipennis*. A-1 = 2 hr old, A-2 = 38 hr old, A-3 = 58 hr old, A-4 = 78 hr old. Regions a–d are addressed in the text.

shown in Fig. 5. Each panel contains all the phenotypes that were represented in the insect colony.

Since all flies assayed were from a single laboratory colony that had been maintained for 30 yr without the introduction of new genetic material, it was presumed that the colony might exhibit a reduced level of genetic variability as compared to natural populations. Consequently, no attempt was made to calculate genotypic frequencies or percent heterozygosity since the values would almost certainly be skewed. The purpose of Fig. 5 is to show that certain enzyme systems can be visualized in individual *C. variipennis* specimens, and that certain of these enzymes can possibly be used in studies of natural populations.

*Results of the enzyme analyses summarized*

*Malic enzyme* (*Me*). No variability could be detected for this enzyme. Each fly assayed exhibited a relatively dark staining band (Fig. 5a).

*Triosephosphate isomerase* (*Tpl*). No variability was detected within the insect colony. Each fly assayed exhibited a faster migrating dark band accompanied by two less intense, more slowly migrating bands (Fig. 5b).

*Hexokinase* (*Hk*). The insect colony exhibited no genetic variability for this enzyme. Three isozymic bands migrated anodally, with the fastest band staining more intensely than the other two bands (Fig. 5c).

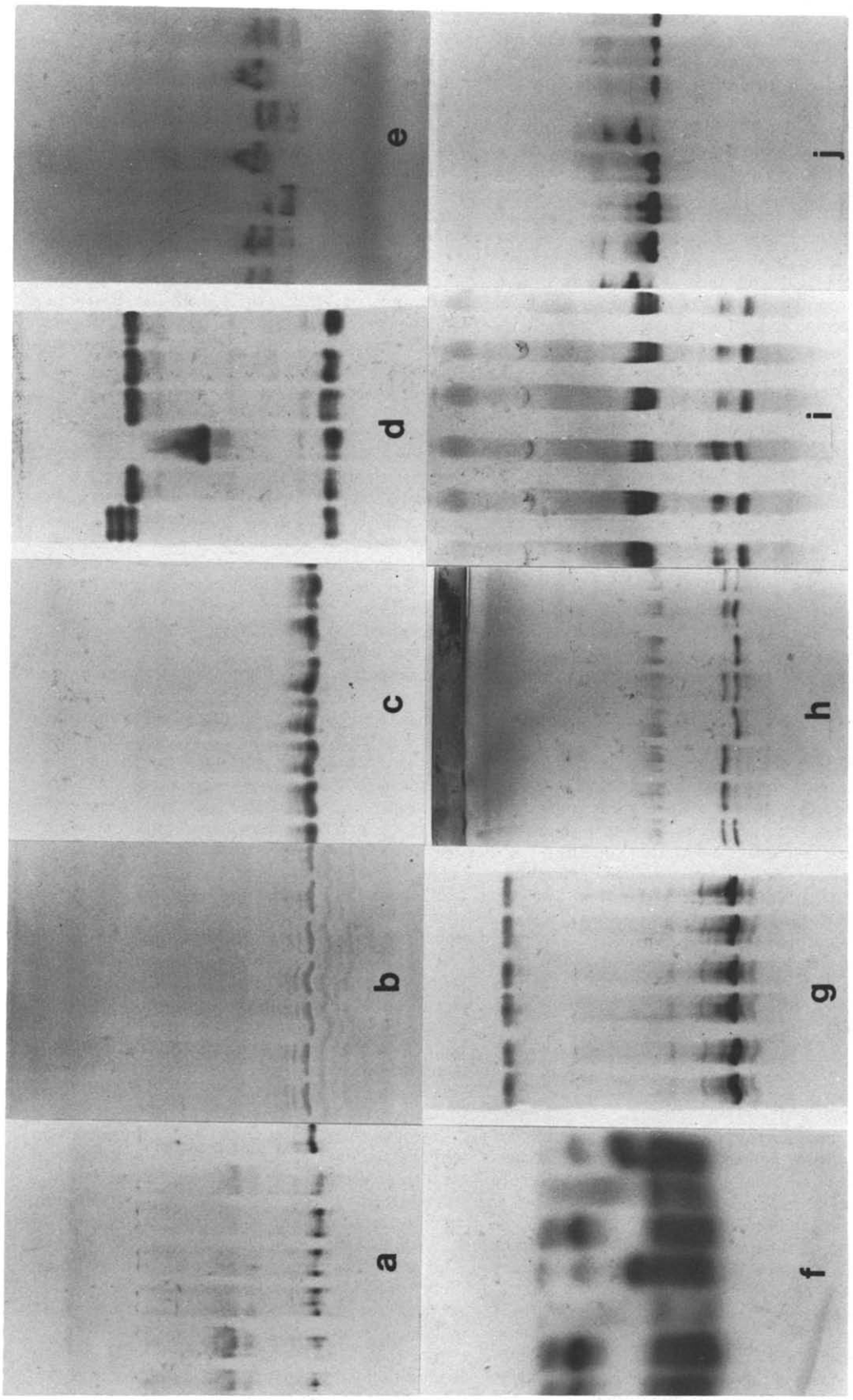


Fig. 5. Isozymes in adult (24–48 hr) female *Culicoides variipennis*. $N = 75$ for each enzyme system. a = malic enzyme, b = triosephosphate isomerase, c = hexokinase, d = phosphoglucoisomerase (upper), phosphoglucomutase (lower), e = 6-phosphogluconate dehydrogenase, f = peptidase, g = malate dehydrogenase (upper), α-glycerophosphate dehydrogenase (lower), h = non-specific esterase, i = aldehyde oxidase, j = isocitrate dehydrogenase. Variability is evident in phosphoglucoisomerase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, peptidase, non-specific esterase and isocitrate dehydrogenase.

*Phosphoglucoisomerase* (*Pgi*). Variability was detected by the cathodally migrating isozymes of Pgi. The well resolved, dark staining bands indicate that a number of electrophoretic patterns may be possible for this enzyme in field collected populations of *C. variipennis*. It appears that more than one locus may be coding for this enzyme since the observed banding patterns correspond with the known quaternary structure of Pgi (Fig. 5d, upper).

*Phosphoglucomutase* (*Pgm*). Two electrophoretic banding patterns were detected for Pgm. The dark staining isozymes migrated anodally and focused close together indicating similar isoelectric points. Both two banded and three banded forms were present within the colony flies. Quite possibly this enzyme system is coded by two loci, one being polymorphic (Fig. 5d, lower).

6-*Phosphogluconate dehydrogenase* (6-*Pgdh*). Variability was detected for 6-Pgdh with three banding patterns exhibited by colony flies (Fig. 5e).

*Peptidase* (*Pep*). Although variability was apparent for Pep, resolution of the electrophoretic bands was relatively poor. Numerous isozymes occupied a wide pH range (approximately pH 5.0–7.5); however, the actual number of bands could not be discerned (Fig. 5f).

α-*Glycerophosphate dehydrogenase* (α-*Gpdh*). Each specimen analyzed revealed an identical electrophoretic pattern for α-Gpdh. A very intense band that migrated anodally was accompanied by at least six minor bands (Fig. 5g, lower).

*Malate dehydrogenase* (*Mdh*). The colony exhibited no variability for Mdh. A single intensely staining band that migrated cathodally was observed from each specimen assayed (Fig. 5g, upper).

*Non-specific esterase* (*Est*). Three electrophoretic banding patterns for Est were revealed. These patterns indicate that there is a single locus controlling Est, that there are two alleles, and that the active form of the enzyme is a monomer (Fig. 5h).

*Aldehyde oxidase* (*Aldox*). Genetic variability within the laboratory colony was not evident for Aldox. The isozymes stained darkly and occupied a relatively wide pH range (Fig. 5i).

*Isocitrate dehydrogenase* (*Idh*). Although variability was evident for Idh, resolution of bands was relatively poor. Intense bands in two positions were detected (Fig. 5j).

## DISCUSSION

Protein components in *C. variipennis* vary at different stages of development. This is in agreement with similar research involving other species of insects (Liu and Dixon, 1965; Wang and Patton, 1968; Lensky, 1971). Unlike much of the earlier work, however, we found the major differences between life stages to be quantitative, with few detectable qualitative changes occurring throughout development.

As indicated in Figs 1–5, there were relatively gradual, yet dramatic, quantitative changes in at least four regions of the gel (regions a, b, c and d). The protein components in these regions increased markedly during the larval and/or early pupal stages, gradually decreased during the pupal stage, and reached a lower, but relatively constant, level of activity in the adult insect.

We did not find any cyclical appearance of new proteins associated with the moult of each instar as reported by Steinhauer and Stephen (1959) in *Periplaneta americana* and by McCormick and Scott (1966) in *Locusta migratoria migratorioides*. Similarly, Lensky (1971) reported that during the development of the bee worker (*Apis mellifera* L.) from egg to adult, there were three distinct patterns of proteins: larval, adult and common to all stages. Lensky's findings are in general agreement with results of studies of ontogeny in extracts of homogenized holometabolous insects (Duke and Pantelouris, 1963; Loughton and West, 1965; Chen and Levenbook, 1966) which demonstrated the appearance of specific protein components during the development of an insect.

Many of the quantitative changes that we observed in the developing *Culicoides* follow the same trend as found in *Hyalophora cecropia* (Chefurka, 1953) and *Bombyx mori* (Wyatt *et al.*, 1956). That is, the concentrations rise in the growing larva, reach a peak at some point in the pupa, then taper off to a lower concentration in the adult insect.

It has been generally accepted since the 1950s that the unique features of each life stage of an insect are built from sets of genes that are unique to that stage (i.e. larval sets of genes, pupal sets, and adult sets) (Wigglesworth, 1959). It was also maintained, however, that certain genes (such as those that code for the structural proteins of muscle) are common to all stages of the insect. Investigators since that time have focused their attention on the often minute differences in proteins during the development of an insect. In her excellent review article, however, Willis (1986) reminds us that metamorphosis is a continuing developmental process, and that individual tissues must obtain the necessary information for "playing out their roles" very early in embryonic development.

Our studies indicate that nearly all of the protein components that could be detected by SDS–PAGE were electrophoretically identical in the egg, larva, pupa, and adult stages. Assuming that the proteins resolved by SDS–PAGE are representative of the proteins necessary for developmental changes, this is evidence that the same proteins are present in all life stages, and that under the right sets of metabolic conditions the same proteins can be used to construct the necessary tissue and mediate the appropriate metabolic processes unique to the particular life stage.

Insect colonies that are maintained for many years without the introduction of new genetic material frequently display traits that are characteristic of inbred populations. These traits include reduced egg viability, adult insects that are smaller in overall size and exhibit a shortened life span, and various genetic anomalies. Electrophoretic analyses of inbred populations frequently indicate a high degree of homozygosity for the loci examined (Nunamaker and Wilson, 1981b).

The specimens used in this study were obtained from a population that had been colonized in 1957. The colony was maintained in a laboratory, in isolation, and on at least one occasion insect production

was reduced from an average of 34,000 adult flies per week to approximately 200 flies per week (Hunt, personal communication). Consequently, one might expect this colony to exhibit signs of inbreeding. On the contrary, at least five of the enzymes that could be assayed electrophoretically exhibited some degree of variability. However, since the colony had been maintained for so many years without the introduction of new genetic material, we surmised that the enzyme phenotypes which we detected are an underestimate of what may actually occur in natural populations. Hence, we made no attempt to calculate allelic frequencies, genotypic frequencies or percent heterozygosity since those data would be of little value. Our primary intent was to determine whether individual flies produced enough of the enzymes in question to be detected by using electrophoretic techniques and specific histochemical stains. Since a number of enzymes can be visualized from individual adult specimens, it would appear that electrophoresis may be a useful tool in identifying *Culicoides* to the species level. Furthermore, if certain of these enzymes (e.g. non-specific Est, Idh, Pep, Pgm, Pgi and 6-Pgdh) exhibit a relatively high degree of genetic variability under field conditions, isoelectric focusing could serve as a useful tool in the taxonomy of the *variipennis* complex. Finally, IFPAG may provide a means for identifying the genotypes of *C. variipennis* that are "efficient" biological vectors of blue-tongue virus disease.

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